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Bridging the NFAT and NF-κB Families: NFAT5 Dimerization Regulates Cytokine Gene Transcription in Response to Osmotic Stress

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Summary

The transcription factor NFAT5/TonEBP is evolutionarily the oldest member of the NFAT/Rel family of transcription factors. We show that NFAT5 is uniquely related to NF-κB and is the only member of the Rel/NFAT family to be activated by osmotic stress. Like Rel/NF-κB proteins but unlike the calcium-regulated NFAT proteins, NFAT5 is constitutively dimeric, and dimerization is essential for DNA binding and transcriptional activity. Using dominant-negative proteins that inhibit NFAT5 dimerization, we show that NFAT5 regulates expression of the TNFα and lymphotoxin-β genes in osmotically stressed T cells. Chromatin immunoprecipitation experiments confirm that NFAT5 binds to the TNFα promoter in vivo. We suggest that NFAT5 participates in specific aspects of host defense by upregulating TNF family genes and other target genes in T cells.

Introduction

Exposure of immune cells to increased plasma osmolarity elicits cytokine expression. Peripheral blood mononuclear cells exposed to elevated sodium concentrations show synthesis of the proinflammatory cytokine IL-8 (Shapiro and Dinarello, 1995) and also increased ability to produce IL-1α, IL-1β, IL-8, and TNFα upon lipopolisaccharide (LPS) or serum stimulation (Shapiro and Dinarello, 1997). Clinically, hypertonic stimulation of the immune system may have either beneficial or deleterious consequences. Hypertonic saline treatment after trauma reduces the risk of sepsis, increases T lymphocyte function, and reduces posttraumatic immunosuppression (Junger et al., 1994, 1997). Conversely, patients with chronic renal failure who undergo intensive ultrafiltration by peritoneal dialysis display elevated levels of IL-1, IL-6, IL-8, and TNFα because they are exposed to solutions of 346–485 mOsm/liter (regular plasma levels are 280 mOsm/liter) (Kronfol, 1994; Descamps-Latscha et al., 1995; Nakanishi et al., 1994; Pereira et al., 1994; Smolen et al., 1996). In these patients, T lymphocytes are often found chronically activated (Caruana et al., 1992), and a higher incidence of autoimmune diseases, such as arthritis, is observed (Goldstein et al., 1985; Takayama et al., 1998).

Treatment of cells with hypertonic media induces a genetic program of adaptive cellular responses in which cells synthesize or accumulate small organic osmolytes, including sorbitol, myo-inositol, betaine, and taurine (Burg et al., 1997; Haussinger, 1996; Kwon and Handler, 1995). This response is especially pronounced in cells of the renal inner medulla, which are routinely exposed to variably high osmolarity. Among the proteins induced in response to osmotic stress are aldose reductase (AR), which mediates the synthesis of sorbitol; the betaine transporter (BGT1); the coupled sodium-myo-inositol transporter; the taurine transporter; and certain heat shock proteins (Burg et al., 1997). The regulatory regions of the *AR* and *BGT1* genes have been shown to contain key functional tonicity response elements with the conserved sequence 5′-TGGAAAA (Burg et al., 1997). This site matches exactly the consensus DNA recognition sequence for proteins of the NFAT family of transcription factors (Rao et al., 1997). The protein binding to the tonicity response element (TonE) of the *BGT1* gene, TonEBP, was isolated in a yeast one-hybrid assay (Miyakawa et al., 1999) and shown to be identical in sequence to NFAT5, a protein independently isolated as a member of the NFAT family (López-Rodríguez et al., 1999a). It is not known whether NFAT5 plays a physiological role in expression of genes other than osmoprotective genes in any cell type.

Evolutionarily, NFAT5 is the most ancient member of the NFAT family; the single NFAT-like protein encoded in the *Drosophila* genome is closely related to NFAT5 (Adams et al., 2000), suggesting that NFAT5 diverged from Rel proteins early in evolution. The mammalian genome encodes four other NFAT proteins (NFAT1–4, also termed NFATc1–c4) that are regulated by Ca^{2+} and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Rao et al., 1997; Neilson et al., 2001). NFAT proteins may be regarded as a distant subgroup of the larger family of Rel/NF-κB proteins (Chen et al., 1999; Chytil and Verdine, 1996). NFAT5 is in many respects an outlier; unlike the calcium-regulated NFAT proteins, NFAT5 does not form cooperative complexes with Fos and Jun and shows no trace of a calcineurin binding regulatory domain (López-Rodríguez et al., 1999a).

In this work, we show that NFAT5 uniquely links the NFAT and NFkB/Rel families and regulates the production of specific cytokines in lymphocytes challenged by osmotic stress. NFAT5 resembles NF-κB/Rel proteins in forming a stable dimer in solution in the absence of DNA, and dimerization is obligatory for DNA binding and transcriptional activity. NFAT5 is the only member of the Rel/NFAT family that is activated by hypertonic stimula-

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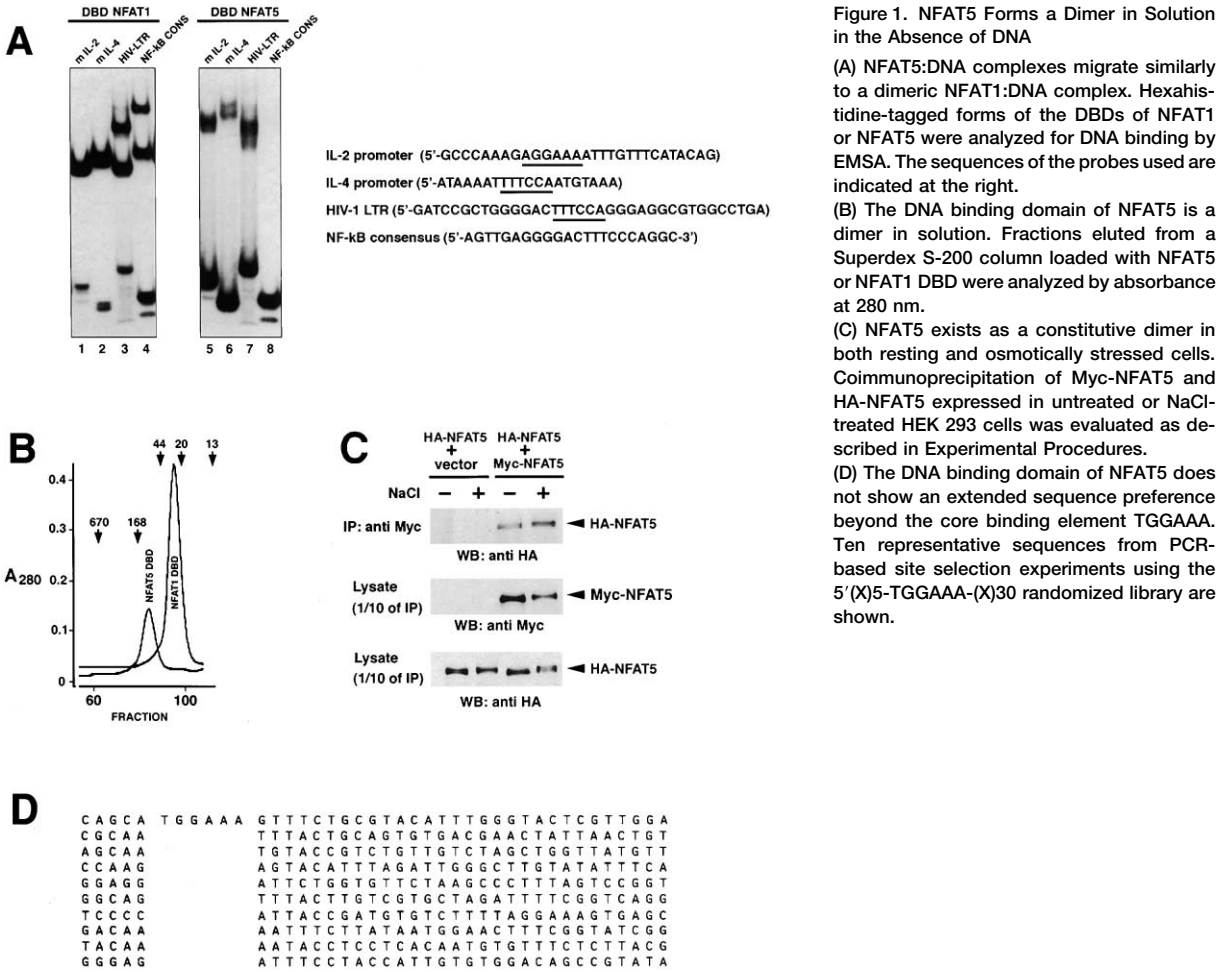

Figure 1. NFAT5 Forms a Dimer in Solution in the Absence of DNA

(A) NFAT5:DNA complexes migrate similarly to a dimeric NFAT1:DNA complex. Hexahistidine-tagged forms of the DBDs of NFAT1 or NFAT5 were analyzed for DNA binding by EMSA. The sequences of the probes used are indicated at the right.

(B) The DNA binding domain of NFAT5 is a dimer in solution. Fractions eluted from a Superdex S-200 column loaded with NFAT5 or NFAT1 DBD were analyzed by absorbance at 280 nm.

(C) NFAT5 exists as a constitutive dimer in both resting and osmotically stressed cells. Coimmunoprecipitation of Myc-NFAT5 and HA-NFAT5 expressed in untreated or NaCl-treated HEK 293 cells was evaluated as described in Experimental Procedures.

(D) The DNA binding domain of NFAT5 does not show an extended sequence preference beyond the core binding element TGGAAA. Ten representative sequences from PCR-based site selection experiments using the 5′(X)5-TGGAAA-(X)30 randomized library are shown.

tion, regulating not only the expression of osmoprotective genes but also *TNF*α and *lymphotoxin*-β (*LT*-β) gene transcription in osmotically stressed T cells. Hypertonic conditions regulate NFAT5 at multiple levels, including protein expression, posttranslational modification, and subcellular distribution. Thus, NFAT5 induces a gene expression program distinct from that elicited by either NF-κB/Rel or calcineurin-regulated NFAT proteins in lymphocytes.

Results

NFAT5 Forms a Dimer in Solution in the Absence of DNA

Despite the similarity of the NFAT5 and NFAT1 DNA binding domains (~43% sequence identity; López-Rodríguez et al., 1999a), NFAT5:DNA complexes migrated significantly more slowly than NFAT1:DNA complexes, using three different oligonucleotide probes (Figure 1A; compare lanes 1 through 3 and 5 through 7). The HIV-1 LTR probe was especially revealing, since this probe can bind either one or two molecules of NFAT1 (Kinoshita et al., 1997; Macian and Rao, 1999); the single band observed with NFAT5 (lane 3) migrated at the position of the NFAT1 dimer complex (lane 7), suggesting strongly that NFAT5 bound DNA as a dimer. Gel filtration experiments confirmed that NFAT5 formed a dimer in solution in the absence of DNA. While the NFAT1 DNA binding domain (DBD1) was monomeric in solution as previously described (Hoey et al., 1995), the NFAT5 DNA binding domain (DBD5) eluted as a stable dimer (Figure 1B).

To determine whether full-length NFAT5 was capable of dimer formation in vivo, we coexpressed HA-tagged and Myc-tagged NFAT5 in human embryonic kidney (HEK) 293 cells. Immunoprecipitation of Myc-NFAT5 led to the appearance of HA-NFAT5 in the immunoprecipitates, indicative of dimer formation (Figure 1C). The level of coimmunoprecipitation was the same under resting and hypertonic conditions (Figure 1C, top panel, compare lanes 3 and 4), demonstrating that NFAT5, like NF-κB/Rel proteins, is constitutively dimeric and that exposure of cells to hypertonic conditions does not change its level of dimer formation.

Our previous site-selection experiments identified a consensus NFAT5 binding sequence, TGGAAA, that was almost identical to that selected by the monomeric protein NFAT1 (López-Rodríguez et al., 1999a). This result seemed surprising, given the dimeric structure of NFAT5. To determine whether NFAT5 would select a palindromic or repeat-containing binding element if presented with a longer randomized DNA sequence, we

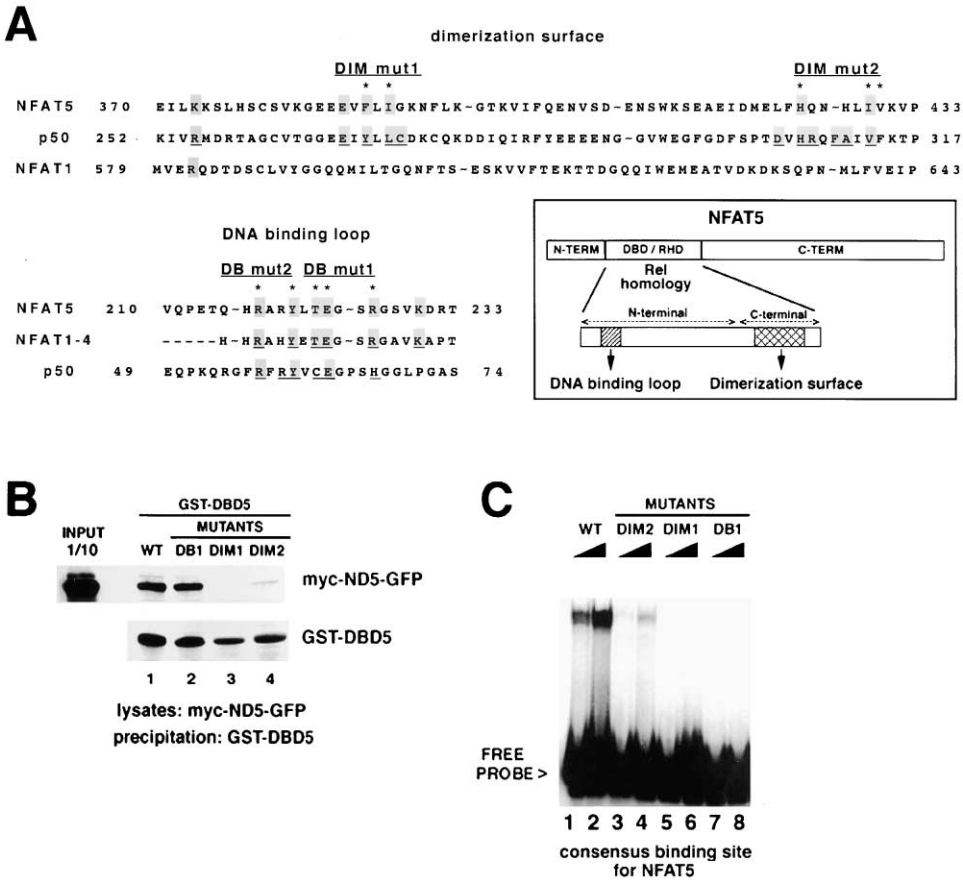

Figure 2. Dimerization Is Required for DNA Binding by NFAT5

(A) NFAT5 conserves Rel family residues involved in DNA binding and dimer formation. The inset indicates the relative positions of mutations in the Rel homology domain (RHD) or DBD of NFAT5. Alignment of the dimer interface (top) and the DNA binding loop (bottom) of NF-κB p50 with the corresponding regions of NFAT5 and NFAT1–4. Residues involved in p50 dimerization and NFAT1–4/NF-κB p50 DNA contact are underlined, and conserved residues are indicated (gray boxes). The residues substituted with alanine in NFAT5 dimerization (DIM) and DNA binding (DB) mutants are indicated with asterisks. NFAT and p50 residues are numbered according to López-Rodríguez et al. (1999a) and Müller et al. (1995).

(B) Dimer formation by wild-type and mutant DBD5 proteins. Lysates of HEK 293 cells expressing Myc-tagged ND5 were tested for binding to wild-type or mutant GST-DBD5. (Top panel) Bound Myc-ND5 was detected by Western blotting with 9E10 (anti-Myc). (Bottom panel) Comparable amounts of GST-DBD5 proteins were used in each case.

(C) DNA binding properties of wild-type and mutant DBD5, analyzed by EMSA.

synthesized two oligonucleotide pools in which a fixed TGGAAA sequence capable of binding NFAT5 was flanked at either its 3′ or 5′ end by randomized 30 bp sequences. Repeated selection of these pools with recombinant NFAT5 DNA binding domain, either in the presence or absence of nuclear extract from high salt-treated cells, did not lead to any discernible selection of preferred adjacent sequences (shown in Figure 1D for the 3′ randomized oligonucleotide pool). These results suggest that the two NFAT5 monomers in a dimer cannot simultaneously make base-specific contacts with adjacent sites in DNA.

Dimerization Is Required for DNA Binding by NFAT5

To identify residues that might contribute to NFAT5 dimerization, we compared the known dimerization interface of p50 NF-κB (Chen et al., 1998b; Ghosh et al., 1995; Müller et al., 1995; Sengchanthalangsy et al., 1999) with the corresponding C-terminal regions of the NFAT1 and NFAT5 DNA binding domains (Figure 2A). The conservation of dimer-forming residues in NFAT5 was significantly more striking than in NFAT1. To determine whether the conserved residues were involved in dimer formation, we generated dimerization mutants DIM1 and DIM2 and DNA binding mutants DB1 and DB2, containing alanine substitutions in the predicted dimerization surface of NFAT5 and in residues known by structural analysis to be DNA contact residues in NFAT and NF-κB (Chen et al., 1998a, 1998b, 1999; Ghosh et al., 1995; López-Rodríguez et al., 1999b; Müller et al., 1995; Zhou et al., 1998) (Figure 2A).

Dimer formation by the mutant proteins was evaluated by comparing the ability of wild-type and mutant GST-DBD5 proteins to bind to a GFP fusion protein, GFP-ND5, expressed in HEK 293 cells (Figure 2B). GFP-ND5 contains the N-terminal region and DNA binding domain of NFAT5 but lacks the long C-terminal region (see Figure 3F). The DNA binding mutants DB1 and DB2 were not impaired for dimerization, since they bound GFP-ND5 as efficiently as wild-type DBD5 (lanes 1 and 2 and

data not shown). In contrast, the dimerization mutant GST-DIM1 was completely incapable of binding GFP-ND5, while GST-DIM2 showed a low level of residual binding (lanes 3 and 4). Thus, the mutated residues contribute to dimer formation, emphasizing the structural similarity between Rel proteins and NFAT5.

Interference with dimer formation impairs DNA binding by NF-κB/Rel proteins (Ryseck et al., 1995). To test whether this was also true for NFAT5, we evaluated DNA binding by the mutant NFAT5 DBDs (Figure 2C). As expected, the DNA binding mutants DB1 and DB2 were unable to bind DNA (lanes 7 and 8 and data not shown). The dimerization null mutant DIM1 showed no DNA binding (lanes 5 and 6), while dimerization mutant DIM2, which showed some residual dimerization, retained a low level of DNA binding (lanes 3 and 4). The complete correspondence between dimer formation and DNA binding indicates that NFAT5, like Rel/NF-κB proteins (Ryseck et al., 1995; Ghosh et al., 1998), binds DNA as an obligate dimer.

Dimerization Is Required for the Transcriptional Activity of NFAT5

To determine whether dimerization is required for the transcriptional function of NFAT5, we took advantage of two established points: (1) NFAT5 is responsive to osmotic stress and is capable of activating reporter plasmids driven by osmotic response elements (OREs), and (2) the C-terminally truncated form of NFAT5, ND5, behaves as a dominant-negative protein in such reporter assays (Miyakawa et al., 1999; Trama et al., 2000).

To map the minimal region of NFAT5 required for dominant-negative activity, we used GFP fusion proteins encoding three truncated versions of NFAT5: ND5, the isolated DNA binding domain of NFAT5 (DBD5), and the minimal dimerization domain (DD5) (see Figures 2A, 3A, and 3F). DD5 corresponds to the C-terminal domain of the Rel homology region which mediates dimer formation in all NF-κB/Rel proteins (Chen et al., 1998b; Ghosh et al., 1995; Müller et al., 1995; Sengchanthalangsy et al., 1999) and contains the dimer interface but not the DNA specificity loop of NFAT5. Smaller truncations were not tested, since they would not encode stable protein domains (Figure 2A). All three truncated proteins showed equivalent levels of dominant-negative activity when expressed in Jurkat T cells, markedly diminishing activation of the ORE-Luc reporter in response to osmotic stress (Figure 3A). In all three cases, dominant-negative activity was specific for the NFAT5-driven ORE reporter and had no effect on reporter activity driven by the cyclosporin-sensitive NFAT proteins or by NF-κB (Figures 3B and 3C). Thus, the minimal dimerization domain of NFAT5 (DD5) suffices for dominant-negative activity.

To confirm that dominant-negative activity reflected interference with dimer formation, we tested the effects of the DBD5 mutants on reporter activity driven by the ORE (Figure 3D). The DNA binding mutant of DBD5 (DB1) inhibited ORE-Luc activity effectively; the DIM1 mutant was ineffective; and the DIM2 mutant, in which dimer formation was impaired but not abrogated, showed intermediate inhibitory activity (Figure 3D and data not shown). All three dominant-negative proteins were expressed at equivalent levels in HEK 293 cells (Figure 3D, right panel). Thus, the inhibitory effect of DBD5 involves interference with dimer formation rather than simple competition for NFAT5 binding sites in DNA (Figure 3F; see Discussion).

The above data suggested that the C-terminal region of NFAT5 possessed transactivation capacity. Indeed, the transactivation function of NFAT5 mapped to its C-terminal region (Figure 3E), suggesting that full-length NFAT5 dimers are transcriptionally active because they possess two transactivation domains, while mixed dimers in which one partner lacks a transactivation domain are transcriptionally inactive (Figure 3F).

NFAT5 Mediates Transcription of Cytokine Genes in Osmotically Stressed T Cells

Hypertonic conditions induce or potentiate the transcription of certain cytokine genes in immune system cells (see Introduction), suggesting that NFAT5 might regulate a broader range of physiological functions than merely the osmoprotective response. To explore this point, we used multiprobe RNase protection assays (RPA) to monitor expression of 30 different cytokine mRNAs in Jurkat T cells exposed to hypertonic conditions (Figure 4 and Experimental Procedures). We reliably observed altered expression of only 2 of these 30 genes: LT-β mRNA was induced in response to added NaCl (Figure 4A), and mRNA encoding the proinflammatory cytokine TNFα was induced in response to combined treatment with NaCl and PMA (Figure 4B). The requirement for combined stimulation is consistent with previous findings that the highest levels of proinflammatory cytokine expression are observed in vivo when osmotic stress is concurrent with microbial invasion (Brauner et al., 1996) and that LPS and osmotic stress synergize to promote cytokine production by peripheral blood leukocytes ex vivo (Shapiro and Dinarello, 1997). On occasion, combined NaCl/PMA treatment induced weak upregulation of IL-8 mRNA compared to PMA stimulation alone, but, because of its variability, this effect was not further pursued. The osmotic stress-dependent induction of TNFα and LT-β mRNAs was observed at 6 hr but not at 2 hr and was not blocked by cyclosporin A (Figure 4 and data not shown).

To determine the involvement of NFAT5 in transcription of these cytokine genes, we used the specific inhibitor GFP-DBD5 (Figure 4). Jurkat cells were transfected with murine CD4 (mCD4) and GFP-DBD5 or GFP alone as a negative control, productively transfected cells were selected for mCD4 expression (Aramburu et al., 1999), and cytokine mRNA levels were analyzed by RNase protection. Overexpression of DBD5 specifically inhibited cytokine gene transcription in response to osmotic stress, without affecting PMA- or PMA/ionomycin-induced gene transcription (Figures 4C and 4D). Thus, DBD5 inhibited LT-β mRNA induction in Jurkat T cells treated by addition of 100 mM NaCl but not in the same batch of transfected Jurkat T cells treated with PMA (Figure 4C). Similarly, DBD5 inhibited PMA/NaCl-stimulated induction of TNFα mRNA but had no effect on PMA/ionomycin-stimulated induction of IL-3 and MIP-1α mRNA in the same batch of cells (Figure 4D). mRNA levels of the constitutively expressed gene *TGF-β* and the housekeeping genes *L32* and *GADPH* were not altered either by the stress treatment or by expression of GFP-DBD5 in the same batch of cells, confirming the specificity of DBD5-

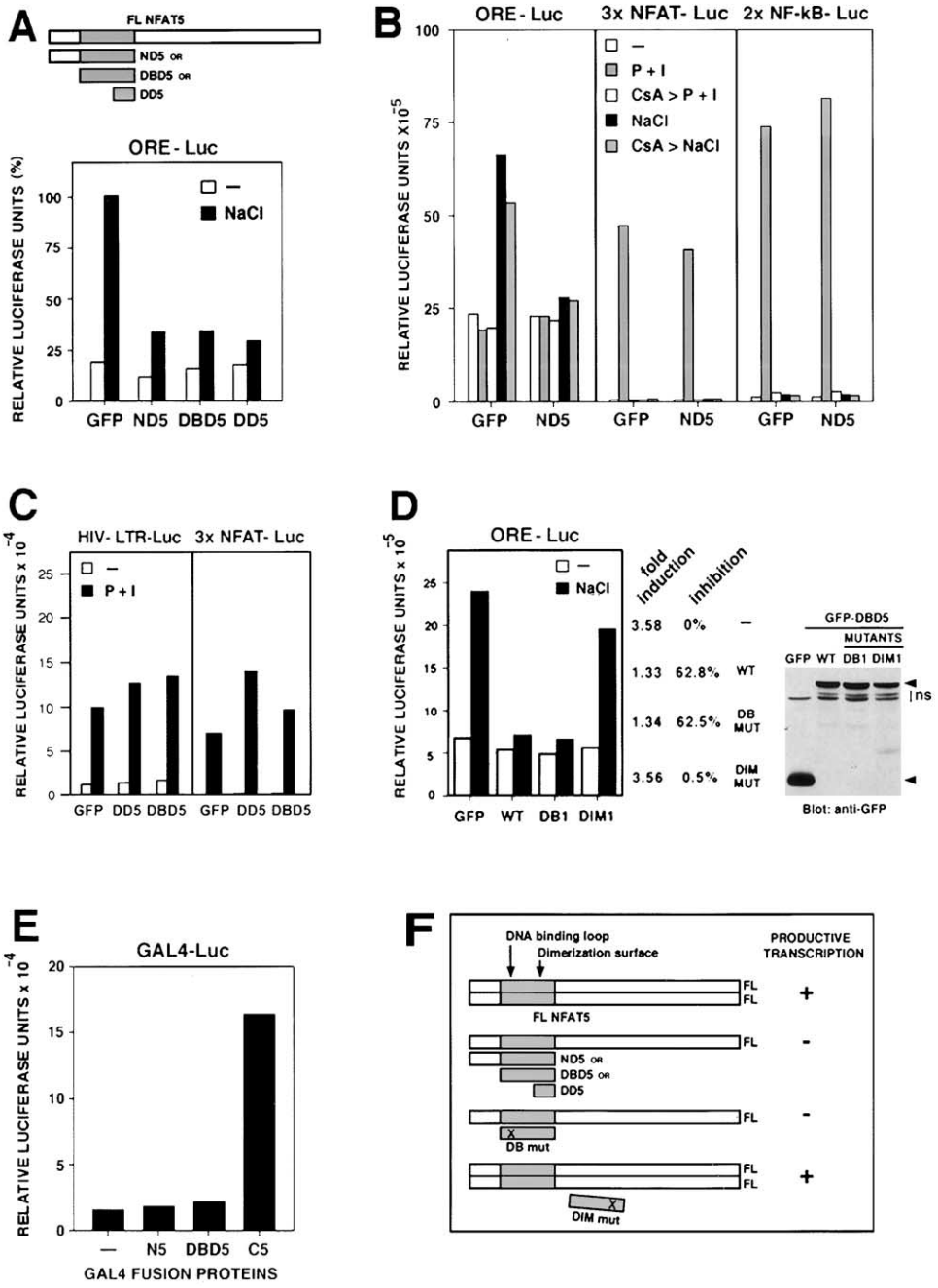

Figure 3. Dimerization Is Required for the Transcriptional Activity of NFAT5

(A) The truncated NFAT5 proteins ND5, DBD5, and DD5 all inhibit reporter activity induced by osmotic stress. Jurkat T cells cotransfected with ORE-Luc and GFP, GFP-ND5, GFP-DBD5, or GFP-DD5 were stimulated by addition of 100 mM NaCl for 6 hr as indicated, and lysates were analyzed for luciferase activity.

(B and C) The dominant-negative NFAT5 proteins ND5, DBD5, and DD5 selectively inhibit reporter activity in response to osmotic stress. Jurkat T cells were transfected with ORE-Luc, HIV-LTR-Luc, NFAT, or NF-κB reporter plasmids and either GFP, GFP-ND5, GFP-DBD5, or GFP-DD5. P, 20 nM PMA; I, 1 μM ionomycin; NaCl, 100 mM NaCl; CsA, 100 nM cyclosporin A.

(D) The inhibitory activity of DBD5 is unaffected by mutations that abrogate DNA binding but eliminated by mutations that abrogate dimer formation. HEK 293 cells were cotransfected with the osmotic stress-responsive reporter plasmid ORE-Luc and expression plasmids encoding GFP, wild-type (WT) GFP-DBD5, or mutant GFP-DBD5 as indicated. Cells were stimulated for 12 hr with added 100 mM NaCl, and cell lysates were analyzed for luciferase activity (left panel). (Right panel) Expression levels of the different GFP-DBD5 proteins in the same experiment.

(E) The C-terminal region of NFAT5 contains a transactivation domain. HEK 293 cells were transfected with a GAL4 reporter plasmid and expression plasmids encoding different regions of NFAT5 fused to the GAL4 DNA binding domain. Cells were lysed 24 hr after transfection, and luciferase activity was analyzed.

(F) The transcriptionally active form of NFAT5 may be a dimer containing two full-length NFAT5 proteins. The model suggests that the dominant-negative proteins ND5, DBD5, and DD5 abrogate productive transcription by competing for dimer formation by endogenous NFAT5. Mutation of residues in the dimerization interface eliminates dominant-negative activity, while mutation of the DNA binding loop does not.

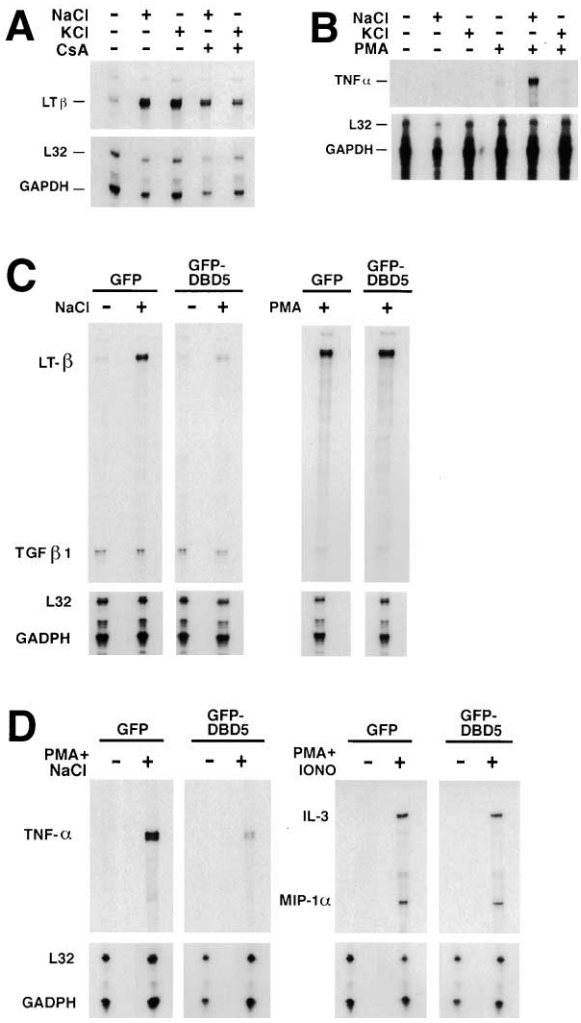

Figure 4. NFAT5 Mediates Cytokine Gene Transcription in Osmotically Stressed T Cells

Jurkat T cells were stimulated for 6 hr as indicated, and cytokine mRNA levels were evaluated by RPA. LT-β, lymphotoxin-β; TGF-β1, transforming growth factor β1; TNFα, tumor necrosis factor α; IL-3, interleukin-3; MIP-1α, macrophage inflammatory protein 1α. (A and B) Osmotic stress modulates transcription of (A) *LT*-β and (B) *TNF*α genes. (C and D) DBD5 selectively inhibits (C) LT-β and (D) TNFα mRNA expression in response to osmotic stress.

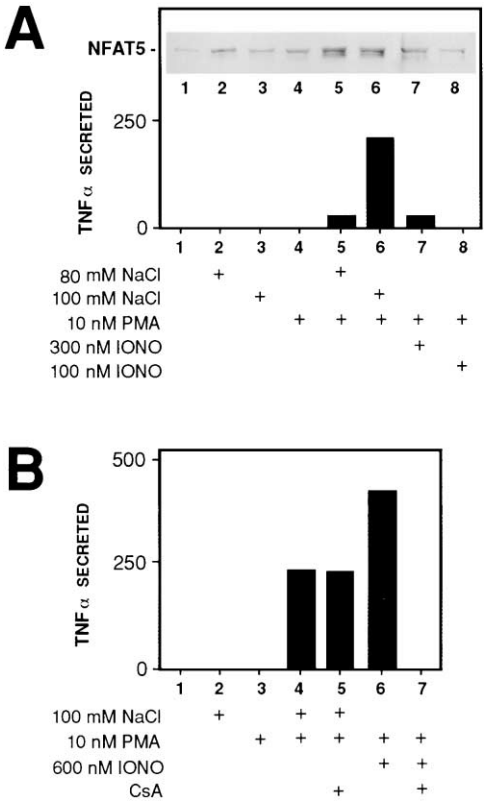

Figure 5. Hypertonic Treatment of Primary Murine T Cells Induces NFAT5 Protein Expression and TNFα Secretion in Parallel

Murine ConA blasts treated for 8 hr with added NaCl, PMA, NaCl plus PMA, or ionomycin plus PMA were tested for TNFα secretion and NFAT5 expression in parallel. Results are shown as pg TNFα produced by 10^7 cells. (A) and (B) show the results of two independent experiments. CsA, cyclosporin A; iono, ionomycin.

mediated inhibition (Figures 4C and 4D). These results indicated strongly that NFAT5 modulates expression of *LT*-β and *TNF*α genes in osmotically stressed T cells.

We tested the role of NFAT5 in cytokine production by primary murine lymphocytes (Figure 5). Unstimulated T cell blasts showed low levels of NFAT5 expression (Figure 5A, lane 1 inset); both NFAT5 levels and TNFα expression were significantly increased by combined stimulation with NaCl and PMA (lanes 5 and 6) and to a lesser extent by stimulation with PMA and 300 nM ionomycin (lane 7). As expected (Goldfeld et al., 1993), TNFα expression in T cells stimulated with PMA and ionomycin was completely abolished by CsA (Figure 5B, compare lanes 6 and 7), while TNFα expression in response to PMA and NaCl was completely insensitive to CsA (compare lanes 4 and 5). The strong correlation between NFAT5 expression and TNFα production seen in these experiments and the fact that osmotic shock does not effectively activate NF-κB or calcium-regulated NFAT proteins (see below) implicate NFAT5 in TNFα production by primary T cells in response to osmotic stress.

NFAT5 Binds In Vivo to Regulatory Regions of the *TNF*α and Aldose Reductase Genes

We used reporter assays to determine whether the proximal promoters of the *TNF*α and *LT*-β genes were responsive to osmotic stress. A reporter plasmid driven by 600 bp of the proximal TNFα promoter was activated ~5-fold by treatment of Jurkat T cells with NaCl and ~8-fold by treatment with NaCl/PMA (Figure 6A), and this activation was inhibited by DBD5 to the same extent as observed for the ORE-Luc reporter in a parallel experiment (data not shown). Thus, the proximal promoter of the *TNF*α gene was a plausible target for direct NFAT5-mediated transcriptional regulation in osmotically stressed T cells. In contrast, the *LT*-β promoter was not activated by osmotic stress (Figure 5B), although it was activated, as expected, by stimulation with PMA (Kuprash et al., 1996), showing that the osmotically respon-

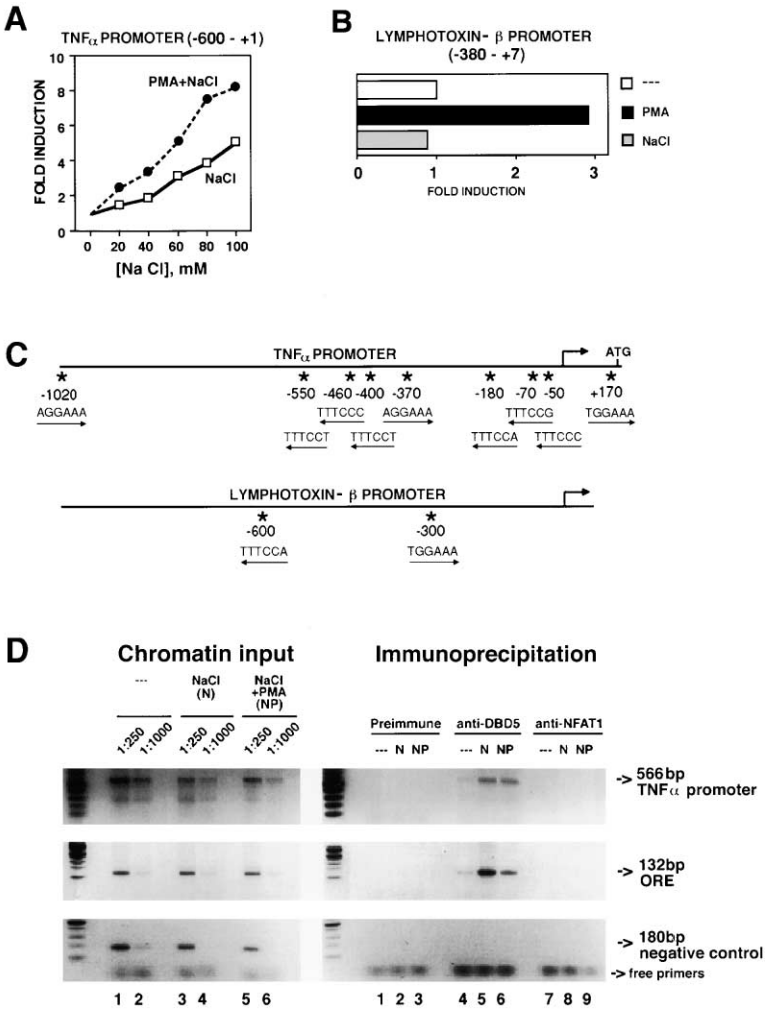

Figure 6. NFAT5 Binds In Vivo to Relevant Regulatory Elements of the Aldose Reductase and *TNF*α Genes

(A) The *TNF*α proximal promoter responds to osmotic stress. Jurkat T cells transfected with a TNFα-Luc plasmid were treated for 6 hr with increasing concentrations of added NaCl, either alone or combined with 20 nM PMA, and lysates were analyzed for luciferase activity. Note that *TNF*α promoter activity was elicited by high salt stimulation alone, while transcription of the endogenous *TNF*α gene requires both high salt and PMA (Figure 4B), suggesting that *TNF*α gene transcription requires not only osmotically responsive factors that bind to the proximal promoter but also PMA-activated nuclear factors that may bind both to the promoter region and to more distal regulatory region(s) of the gene.

(B) The proximal promoter of the *LT*-β gene does not respond to osmotic stress. Jurkat T cells transfected with an LT-β-Luc plasmid were stimulated for 6 hr with 20 nM PMA or 100 mM added NaCl, and luciferase activity was measured in cell lysates.

(C) Schematic representation of the *TNF*α and *LT*-β promoter regions. Consensus sites for NFAT5 binding are indicated by asterisks. Sites located in the −280 to +180 region of the TNFα promoter have been shown to be functional NFAT5 binding sites in vitro.

(D) NFAT5 binds to the aldose reductase enhancer and the *TNF*α proximal promoter in living cells. Jurkat T cells were left untreated (---) or stimulated for 6 hr with NaCl (N) or NaCl plus PMA (NP). After formaldehyde crosslinking, DNA-protein complexes were immunoprecipitated with preimmune serum or antisera against NFAT1 or NFAT5. (Right panels) Presence of the osmotic response element (ORE) of the aldose reductase enhancer, the *TNF*α proximal promoter, and a negative control DNA in the chromatin immunoprecipitates was assessed by PCR. (Left panels) Input chromatin dilutions show comparable levels of target DNA sequences in all the immunoprecipitated samples and also indicate the efficiency of each set of primers in amplifying their specific sequences from different chromatin samples.

sive region of the *LT*-β gene lies outside the −380 bp proximal promoter region.

Visual inspection revealed eight consensus NFAT5 sites in the 600 bp region used for reporter assays (summarized in Figure 6C). Four of these, located in the −280 to +180 bp region, were tested and shown to be capable of binding NFAT5 in vitro (data not shown). Rather than mutating these sites and measuring reporter activity, we tested whether NFAT5 bound in vivo to the TNFα promoter by chromatin immunoprecipitation (ChIP) assays. The ChIP technique involves crosslinking of DNA binding proteins to DNA in living cells, followed by immunoprecipitation and PCR analysis to identify the genomic region bound by the proteins in vivo (Parekh and Maniatis, 1999).

Jurkat T cells were treated with NaCl or NaCl plus PMA under conditions optimal for inducing TNFα mRNA transcription and crosslinked with formaldehyde. Chromatin was prepared, immunoprecipitated with antibodies to NFAT5, and analyzed by PCR for the presence of *TNF*α promoter sequences (Figure 6D, right, top panel), the aldose reductase gene enhancer (ORE) as a positive control (Figure 6D, right, middle panel), or a C-terminal exon of the NFAT5 gene as a negative control (Figure 6D, bottom panel). This analysis revealed specific in vivo binding of NFAT5 to the *TNF*α proximal promoter and the aldose reductase enhancer in response to hypertonic stimulation, either alone or in combination with PMA (Figure 6D, right, top and middle panels, lanes 5 and 6). Weak binding of NFAT5 to both regulatory regions was detected in untreated cells (Figure 6D, right, middle and bottom panels, lane 4); this is likely due to the slight hypertonicity of cell culture media relative to plasma, which results in NFAT5 being present and presumably activated in the nucleus of many nominally resting cells (see below). These results show clearly that NFAT5 binds in vivo to two genomic regions established to be osmotically responsive: the aldose reductase enhancer and the proximal *TNF*α promoter.

NFAT5 Is Regulated at Multiple Levels by Osmotic Stress

We investigated the mechanism of NFAT5 regulation in Jurkat T cells and in the untransformed, IL-2-dependent

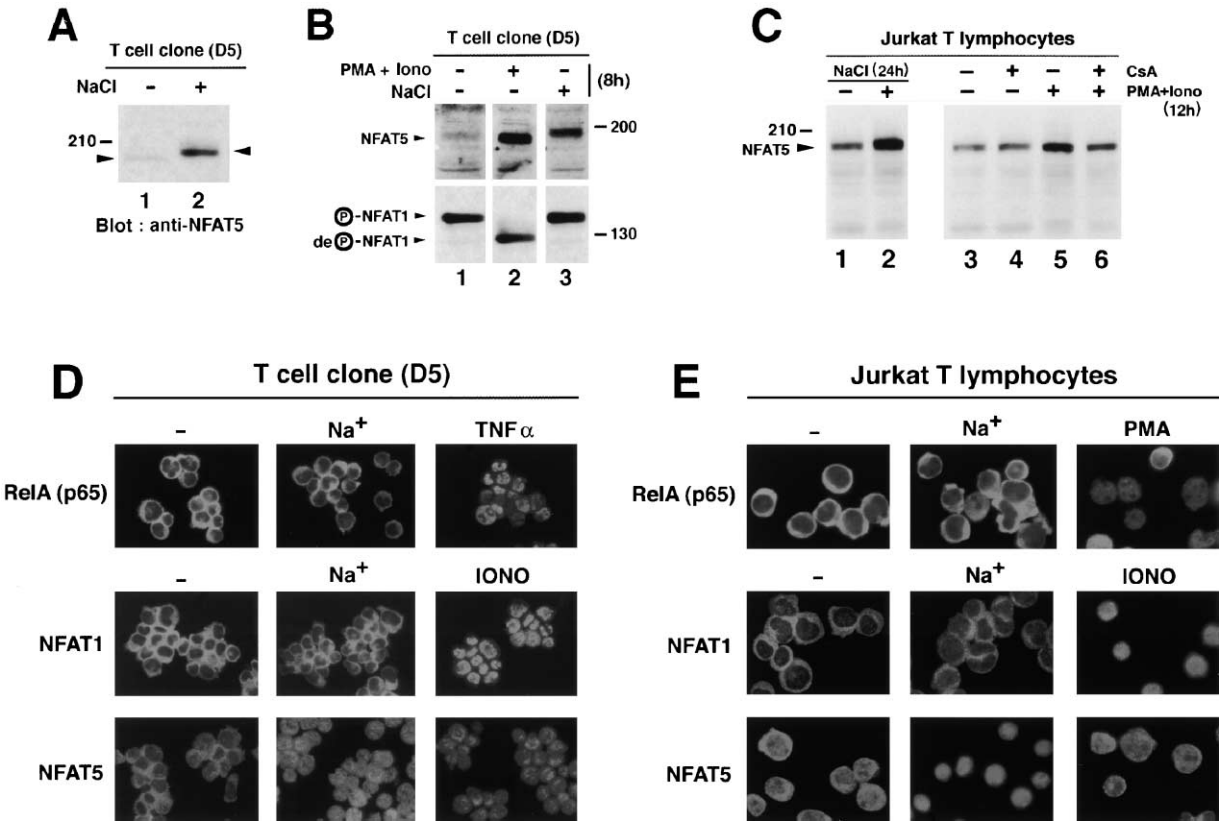

Figure 7. NFAT5 Is Regulated at Multiple Levels by Osmotic Stress

(A–C) Hypertonic conditions upregulate NFAT5 expression and alter its migration in SDS gels. D5 T cells (A and B) or Jurkat T cells (C) were left untreated or stimulated with NaCl or PMA/ionomycin in the presence or absence of CsA as indicated. Cell lysates were analyzed by Western blotting with an anti-NFAT5 antibody. In (B), the same blot was reanalyzed with an antibody to NFAT1 (bottom panel). Arrowheads indicate the positions of NFAT5 and the phospho and dephospho forms of NFAT1. Migration of molecular weight markers is indicated.

(D and E) RelA (p65 NF-κB) and NFAT1 are not activated by osmotic stress. Subcellular localization of transcription factors was detected by immunocytochemistry of D5 T cells (D) or Jurkat T lymphocytes (E) as described in Experimental Procedures.

murine Th1 cell clone D5 (Figure 7). In both cell types, hypertonic stimulation had three striking effects: it altered the posttranslational modification, increased protein expression levels, and altered the subcellular distribution of NFAT5. NFAT5 from osmotically stressed cells migrated with significantly slower mobility than NFAT5 from untreated cells; this effect was reversed by treatment with alkaline phosphatase and not affected by preincubation with CsA (Figures 7A–7C and data not shown). Moreover, osmotically stressed cells showed a marked increase in NFAT5 protein levels (Figures 7A–7C) that was insensitive to CsA (data not shown); the similar increase in protein expression observed in PMA/ionomycin-stimulated cells was suppressed by CsA (Figure 7C, lanes 5 and 6). Finally, NFAT5 was largely (but not completely) cytoplasmic in both D5 and Jurkat T cells under resting conditions but became significantly nuclear after 2–3 hr of exposure to hypertonic conditions (Figures 7D and 7E). There is some variability among different cell lines: a second subline of Jurkat cells displayed predominantly nuclear NFAT5 even under resting conditions, and both the HeLa epithelial human cell line and the murine T lymphocyte clone Cl.7W2 had mostly nuclear NFAT5 with a moderate level of cytoplasmic localization (López-Rodríguez et al., 1999a). This variability may reflect differential sensitivity of different cell lines to cell culture media, which are generally slightly hypertonic relative to plasma (310 versus 280 mOsms).

NFAT1 and RelA Are Not Activated by Osmotic Stress

To rule out involvement of NF-κB and calcium-regulated NFAT proteins in cytokine gene expression in response to osmotic stress, we examined their behavior in cells exposed to hypertonic conditions. ChIP assays failed to show detectable association of NFAT1 with the aldose reductase or TNFα regulatory regions in NaCl- or NaCl/PMA-treated cells, under conditions where NFAT5 binding was clearly apparent (Figure 6D); the anti-NFAT1 antiserum used in this experiment efficiently precipitates NFAT1 bound to known NFAT1-dependent regulatory regions in the *IL-4* and *IFN-γ* genes (Agarwal et al., 2000). Likewise, NFAT1 was not dephosphorylated nor altered in its expression levels in hypertonically stimulated D5 cells (Figure 7B, bottom panel, lanes 1 and 3). Moreover, in several cell lines including the D5 T cell clone, Jurkat T lymphocytes, Cl7W2 T cells, and HeLa cells, Rel A (p65 NF-kB) and NFAT1 did not translocate to the nucleus under hypertonic conditions, which resulted in

nuclear accumulation of NFAT5 (Figures 7D and 7E, top and middle panels, and data not shown). Together, these results implicate NFAT5 but not Rel/NF-κB or calcium-regulated NFAT proteins in cytokine gene transcription in osmotically stressed T cells.

Discussion

NFAT5 Shows Features of Both the NFAT and NF-κB/Rel Families of DNA Binding Proteins

NFAT5 shows intriguing similarities to both the NFAT and NF-κB/Rel families of proteins, resembling NFAT1–4 in its DNA binding specificity and NF-κB/Rel in forming a stable dimer in the absence of DNA and binding DNA as an obligate dimer. Mutation of conserved residues in the dimer interface impairs DNA binding by Rel proteins as well as NFAT5, implying similar dimer architectures in each case. Curiously, the NFAT5 dimer selects monomeric DNA binding sites (A/TGGAA) that are identical to those selected by the monomeric NFAT1–4 proteins but very different from the palindromic elements bound by NF-κB/Rel (GGGRNNYYCC) (López-Rodríguez et al., 1999a; Miyakawa et al., 1999; Rao et al., 1997). This implies that NFAT5 monomers are oriented in the dimer such that they cannot simultaneously select adjacent binding sites in a short randomized oligonucleotide. As shown for the glucocorticoid receptor dimer (Luisi et al., 1991), one monomer might make base-specific interactions with DNA, while the other makes backbone contacts. Alternatively, the TGGAAA sequences bound by the individual monomers in a dimer might be separated by >30 basepairs, a lower limit imposed by the length of the randomized sequence used in our site-selection experiments, and NFAT5 might link them in a *cis*-regulatory loop, as postulated for the lambda repressor (Bell et al., 2000). In any case, the configuration of transcriptionally active complexes containing NFAT1–4 and NFAT5 must differ, since NFAT5 does not activate reporters driven by tandem copies of conventional NFAT sites, in either PMA/ionomycin-stimulated or osmotically stressed T cells (Trama et al., 2000, and this report).

Dimer formation is also required for transactivation by NFAT5, since the dominant-inhibitory effect of truncated NFAT5 proteins depends on their ability to dimerize but not on their ability to bind DNA. The most likely explanation is that a dimer of full-length NFAT5 is required for productive gene transcription: mixed dimers containing only one C-terminal transactivation domain, formed with the truncated dominant-negative proteins and endogenous NFAT5, are not able to transactivate even if they are capable of binding to DNA (Figure 3F). Thus, targeting the dimerization interface of NFAT5 is a feasible and selective strategy for inhibiting NFAT5 function without affecting transcription mediated by conventional NF-κB/Rel dimers or Ca^{2+}-regulated NFAT proteins.

NFAT5 Is Selectively Activated by Multiple Mechanisms in Response to Osmotic Stress

NFAT5 is the only member of the extended Rel/NFAT family that responds to osmotic stress. NF-κB/Rel proteins respond to a variety of stimuli, including T lymphocyte mitogens, proinflammatory cytokines, DNA-damaging conditions (UV and oxidative stress), and a number of viruses (Ghosh et al., 1998), but they are not activated in response to hypertonic conditions (Wesselborg et al., 1997; this report). The calcium-activated NFAT proteins are regulated primarily by a phosphorylation-dependent cycle of nuclear import and export, controlled by the balance between the activities of calcineurin and NFAT kinases (Okamura et al., 2000), and neither the subcellular distribution nor the transcriptional activity of NFAT1 is detectably altered by osmotic stress.

We have shown that NFAT5 activity is regulated by multiple mechanisms in osmotically stressed T cells. One mechanism involves posttranslational modification which includes but may not be limited to phosphorylation over the basal level observed in resting cells (López-Rodríguez et al., 1999a). The kinases that mediate the inducible phosphorylation do not seem to be the stress-responsive MAP kinases p38 or JNK, since overexpression of constitutively active versions of their upstream MAP kinases MKK3 and MKK6 had no effect on NFAT5 expression or function (C.L.-R., unpublished data). A second mechanism is increased protein expression that can be induced by two distinct signaling pathways: a calcineurin-independent pathway activated by osmotic stress and a calcineurin-dependent pathway activated by mitogens such as anti-CD3, ConA, or PMA/ionomycin stimulation (also reported by Trama et al., 2000). Finally, a third mechanism involves NFAT5 translocation to the nucleus in response to hypertonic stimulation. This mechanism is not readily demonstrated, since most cell culture media are hypertonic relative to plasma, leading to some degree of constitutive nuclear localization and activation in most cultured cells. Moreover, transformed cell lines show a strong tendency to constitutive nuclear localization of NFAT5 (López-Rodríguez et al., 1999a; C.L.-R., unpublished results), and hypertonic treatment of primary T cells simultaneously induces NFAT5 protein expression and its nuclear localization.

NFAT5 Mediates Cytokine Gene Transcription in Response to Osmotic Stress

In addition to the standard osmoprotective responses documented in kidney cells, NFAT5 induces the transcription of at least two cytokine genes, *LT*-β and *TNF*α, in osmotically stressed T cells. What is the significance of cytokine production under these conditions? While lymphocytes in a healthy animal are not generally exposed to hypertonic conditions, there are many pathological conditions in which this may occur. Extensive burns, which directly expose the patient to invading pathogens, elevate plasma osmolality dramatically (as high as 430 mOsm/kg) (Inglis et al., 1995); similarly, pathogens of the digestive tract trigger high fever, sweating, and diarrhea, resulting in profound dehydration and increased plasma osmolality (Meyers, 1994). From an evolutionary standpoint, any enhancement of immune responses under these conditions would result in improved survival. NFAT5 may also contribute to inflammatory and autoimmune disease under conditions of elevated plasma osmolarity, for instance, the high incidence of autoimmune arthritis observed in patients undergoing dialysis (Goldstein et al., 1985; Takayama et al., 1998).

Does NFAT5 influence the immune response under physiological conditions? NFAT5 may contribute to the organogenesis and apoptosis mediated by TNF family proteins, including TNFα and lymphotoxin-β (Nishikawa et al., 2000; Screaton and Xu, 2000; Locksley et al., 2001). A further role is suggested by the fact that, throughout evolution, proteins of the Rel/NF-κB/NFAT family have had critical functions in immune defense. In mammals, NF-κB/Rel proteins mediate inflammatory responses and cellular defenses against pathogens, injury, and stress (Ghosh et al., 1998), while calcium-regulated NFAT proteins are involved in the induction of a large number of activation-associated genes and preserve an appropriate balance of Th1- and Th2-type cytokines (Kiani et al., 2000). The *Drosophila* genome encodes three bona fide Rel proteins which mediate responses to microbes (Dorsal, Dif, and Relish; Imler and Hoffmann, 2000), as well as one protein with an NFAT-like Rel domain which shows significant sequence identity to human and mouse NFAT5 (55%). Potentially, NFAT5-mediated gene transcription is the endpoint of an evolutionarily conserved signaling pathway that has an important function in innate immune responses or other immune defenses.

Experimental Procedures

Plasmids

The reporter plasmids 3x-NFAT-Luc (Hedin et al., 1997), 2x-NF-κB-Luc (Mercurio et al., 1997), HIV-LTR-Luc (Macian and Rao, 1999), TNFα-Luc (López-Rodríguez et al., 1999a), and GAL4-Luc (Chakravarti et al., 1996) have been described. The osmotic stress-responsive luciferase reporter plasmid ORE-Luc, which incorporates 132 bp of the human aldose reductase gene enhancer containing three consensus binding sites for NFAT5 (Ko et al., 1997; López-Rodríguez et al., 1999a), was made by PCR amplifying genomic DNA (forward primer, 5′-TTACATGGAAAAATATCTGGGCTAG-3′; reverse primer, 5′-CTGGTAGTGACTCAAGCAC-3′) and cloning the resulting PCR fragment 5′ of the minimal SV40 promoter in the luciferase reporter plasmid pGL3 (Promega). The human lymphotoxin-β reporter LT-β-Luc was made by PCR amplifying 380 bp of the human *Lt*-β promoter immediately 5′ of the translation start site (forward primer, 5′-CCAG CACCTACCTTGAGGGTATAG-3′; reverse primer, 5′-TGAGACTGA ACCAGAGCCAGAGC-3′) and subcloning the resulting fragment into pGL3p (Promega) from which the SV40 promoter had been deleted.

The bacterial expression plasmid encoding GST-DBD5 (amino acids [aa] 175 to 471 of NFAT5a) and the mammalian expression plasmids encoding isoform a of human NFAT5 and its C-terminally truncated derivative ND5 have been described (López-Rodríguez et al., 1999a, 1999b). The expression plasmid encoding the dimerization domain of NFAT5 (DD5-GFP) was generated by subcloning a fragment encoding amino acids 362 to 471 of NFAT5 C-terminal to the GFP in the pEGFP N1 plasmid (Clontech). Expression plasmids encoding GAL4 fusion proteins with the DNA binding domain of GAL4 N-terminal to NFAT5 aa 1–190 (N-terminal region), aa 172–471 (DBD), and aa 458–1456 (C-terminal region) were generated using the pM plasmid (Clontech). All constructs were confirmed by sequencing.

To monitor gel filtration behavior, a Superdex S-200 column (Amersham Pharmacia Biotech) was loaded with recombinant DBDs of NFAT5 (0.5 mg/ml) or NFAT1 (0.5–5 mg/ml) in 2 ml of buffer containing 10 mM Tris (pH 8.0), 100 mM NaCl, and 0.5 mM EDTA. The flow rate was 1.0 ml/min. Fractions were analyzed by absorbance at 280 nm to determine their protein concentration.

Mutagenesis and Evaluation of Mutant Proteins

Mutated versions of the NFAT5 DBD containing alanine substitutions as follows were constructed by PCR-mediated mutagenesis with *Taq* polymerase (Perkin Elmer): DNA binding mutant 1 (DB1), T222, E223, and R226; DNA binding mutant 2 (DB2), R217, and Y220; dimerization mutant 1 (DIM1), F388, and I390; dimerization mutant 2 (DIM2), H424, I429, and V430. PCR products were subcloned into pGEX 6P-1 (Pharmacia) and the presence of only the desired mutations was confirmed by DNA sequencing. GST-deleted versions of the mutant proteins were generated by cutting with PreScission protease (Pharmacia). For mammalian expression, wild-type and mutant DBD5 were expressed as GFP fusion proteins with GFP at their amino terminus by subcloning into pEGFP N1 (Clontech). Electrophoretic mobility shift assays (EMSAs) with bacterial proteins were performed as previously described, using an oligonucleotide containing a consensus NFAT5 binding site (López-Rodríguez et al., 1999a). To evaluate dimerization, lysates were prepared from HEK 293 cells expressing Myc-tagged ND5, and 200 μl of lysate was incubated for 60 min at 4°C with ~10 μg of wild-type or mutant GST-DBD5 bound to glutathione-Sepharose beads (see below, Immunoprecipitation). After washing four times with lysis buffer, bound ND5 protein was detected by Western blotting using monoclonal antibody 9E10 (Boehringer Manheim) to the Myc epitope tag.

PCR-Based Site Selection

PCR-based site selection and amplification of DNA binding sites was performed as previously described (López-Rodríguez et al., 1999a), with the DNA binding domain of NFAT5 in the presence of 15 μg of nuclear extracts from salt-treated HEK 293 cells. Two randomized double-stranded DNA libraries were used: 5′(X)5-TGGAAA-(X)30 and 5′(X)30-TGGAAA-(X)5.

Cell Culture, Transfections, and Reporter Assays

The D5 (Ar-5) T cell clone was cultured as described previously (Agarwal et al., 2000). Jurkat T leukemia cells and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 mM HEPES, and 2 mM L-glutamine, and transfected as previously described (López-Rodríguez et al., 1999a). To avoid the presence of pyrogens, all hypertonic treatments were performed using an autoclaved 5 M NaCl stock solution filtered through a 0.22 μm size exclusion filter. At 24 hr after transfection, cells were stimulated by addition of final 20–175 mM NaCl, 1–2 μM ionomycin (Calbiochem), and 20 nM phorbol myristate acetate (PMA) (Calbiochem). Where indicated, cells were preincubated with the calcineurin inhibitor cyclosporin A (CsA; 1 μM) for 15 min at 37°C prior to stimulation. At 6–8 hr after stimulation, cells were harvested, and luciferase activity was assessed as described (López-Rodríguez et al., 1999a). Luciferase values were normalized to an independent reporter (*Renilla* luciferase). All experiments were performed at least twice, and a representative experiment is shown.

Immunoprecipitations

HEK 293 cells were cotransfected with plasmids encoding HA-tagged and Myc-tagged full-length NFAT5 or with the HA-NFAT5 plasmid together with a plasmid expressing the Myc epitope alone (López-Rodríguez et al., 1999a). At 24 hr later, cells were left untreated or were stressed by addition of final 100 mM NaCl to cell culture media for 12 hr. Cells were lysed in 50 mM HEPES (pH 7.4), 110 mM NaCl, 1% NP-40, 10 mM NaPPi, 20 mM β-glycerophosphate, 1 mM sodium-orthovanadate, 1 mM iodoacetamide, and protease inhibitors as described (López-Rodríguez et al., 1999a). Whole-cell lysates were immunoprecipitated with 9E10 anti-Myc antibody and GammaBind Plus Sepharose (Pharmacia) for 3 hr at 4°C. Immunoprecipitates were washed four times in ice-cold lysis buffer, and bound proteins were detected by Western blotting with anti-HA (12CA5; Boehringer Manheim) and anti-Myc antibodies.

Immunocytochemistry

Jurkat T lymphocyte cells or the T cell clone D5 was incubated for 2–3 hr with 100 mM NaCl; for 30 min with 1 μM ionomycin in medium supplemented with extra 2 mM $CaCl_2$; for 1 hr with 50 nM PMA; or for 20 min with 50 ng/ml of mouse recombinant TNFα, followed by centrifugation in a cytospin (Shandon; 3 min/500 rpm) onto coverslips coated with poly-L-lysine (Sigma; 0.01% w/v). Cells were fixed in 3% paraformaldehyde for 20 min at room temperature, permeabilized by washing three times in wash buffer (1× PBS, 0.5%

NP-40), and nonspecific binding was blocked by incubation with wash buffer plus 10% FCS for 1 hr at room temperature. Antibody incubations (1–2 hr, room temperature) were with anti-67.1 (1:1000) for NFAT1 (López-Rodríguez et al., 1999a); anti-N-terminal of NFAT5 (1:500) (López-Rodríguez et al., 1999a); or sc-372 (Santa Cruz Biotechnology) (1:1000) anti-RelA(p65), followed by Cy-3 conjugated sheep-anti-rabbit IgG (Sigma).

Hypertonic Stimulation of Primary Murine T Cells

T cell blasts were prepared by treating BALB/c (Taconic; 5–6 weeks) spleen cells with 2.5 μg/ml ConA and 20 U/ml IL-2 for 2 days, followed by resting in IL-2-containing medium for 12–18 hr. Cells recovered at the end of this period (100% $CD3^+$, ~40% $CD4^+$, 60% $CD8^+$) were incubated (6×10^6 in 2 ml) for 8 hr with added NaCl, PMA, NaCl plus PMA, or ionomycin plus PMA. TNFα secretion and NFAT5 expression were evaluated in parallel in the same cells by ELISA on 100 μl of cell supernatants (PharMingen) and Western analysis of cell lysates, respectively.

RNase Protection Assay

Jurkat cells cotransfected with murine CD4 (mCD4) and GFP or GFP-DBD5 (0.75 μg/10^6 cells) were selected (>90% of $mCD4^+$ cells were also GFP positive); stimulated as indicated by addition of 100 mM NaCl, 50 mM KCl, 20 nM PMA, 1 μM ionomycin, and/or 100 nM CsA for 6 hr; and evaluated for cytokine mRNA expression as described (Aramburu et al., 1999). The human multicytokine template sets used were hCK1 (IL-5, IL-4, IL-10, IL-14, IL-15, IL-9, IL-2, IL-13, and IFNγ), hCK3 (TNFβ, LT-β, TNFα, IFNγ, IFNβ, TGF-β3, TGF-β2, and TGF-β1), hCK5 (RANTES, Lymphotactin, IP-10, MIP-1β, MCP-1, IL-8, and I-309), and a custom-made template set containing probes for FasL, IL-3, TNFα, GM-CSF, MIP-1α, and BCL-2. Cytokine mRNA levels were normalized to levels of housekeeping genes *L32* and *GAPDH*.

Chromatin Immunoprecipitation Assays

ChIP analyses were carried out essentially as described (Parekh and Maniatis, 1999). Unstimulated or stimulated (6 hr, by adding 100 mM NaCl or 100 mM NaCl plus 20 nM PMA) Jurkat T cells (2×10^8), were crosslinked for 20 min at 4°C, using 11% formaldehyde. Nuclei were isolated, sonicated, and DNA-protein complexes were purified by CsCl gradient centrifugation for 40 hr. Chromatin was immunoprecipitated using a a cocktail of anti-NFAT1 antibodies (Agarwal et al., 2000) or a rabbit polyclonal sera that recognizes NFAT5 (López-Rodríguez et al., 1999a). Samples were treated with RNase A and proteinase K, and, following reversal of the crosslinks and organic extraction, they were precipitated. The presence of immunoprecipitated DNA sequences was assayed by semiquantitative PCR. The primers used were as follows: human aldose reductase enhancer (ORE): forward primer, 5′-TTACATGGAAAAATAT CTGGGCTAG-3′, and reverse primer, 5′-CTGGTAGTGACTCAAG CAC-3′, 132 bp product. Human *TNF*α promoter: forward primer, 5′-AGTGAAAGAGCCTCCAGGACCTC-3′, and reverse primer, 5′-CCA CTGACTGATTTGTGTGTAGGACC-3′, 566 bp product. Negative control (NFAT5 coding region): forward primer, 5′-GTTGCCATGCA GAGTAACTCT-3′, and reverse primer, 5′-CATTGGATTTTGATTTG GGTTGAATATCCTG-3′, 180 bp product.

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References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. (2000). The genome sequence of Drosophila melanogaster. Science *287*, 2185–2195.

Agarwal, S., Avni, O., and Rao, A. (2000). Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. Immunity *12*, 643–652.

Aramburu, J., Yaffe, M.B., Lopez-Rodriguez, C., Cantley, L.C., Hogan, P.G., and Rao, A. (1999). Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. Science *285*, 2129–2133.

Bell, C.E., Frescura, P., Hochschild, A., and Lewis, M. (2000). Crystal structure of the lambda repressor C-terminal domain provides a model for cooperative operator binding. Cell *101*, 801–811.

Brauner, A., Hylander, B., and Wretlind, B. (1996). Tumor necrosis factor-α, interleukin-1β, and interleukin-1 receptor antagonist in dialysate and serum from patients on continuous ambulatory peritoneal dialysis. Am. J. Kidney Dis. *27*, 402–408.

Burg, M.B., Kwon, E.D., and Kultz, D. (1997). Regulation of gene expression by hypertonicity. Annu. Rev. Physiol. *59*, 437–455.

Caruana, R.J., Leffell, M.S., Lobel, S.A., Campbell, H.T., and Cheek, P.L. (1992). Chronic T-lymphocyte activation in chronic renal failure: a study of hemodialysis, CAPD and pre-dialysis patients. Int. J. Artif. Organs *15*, 93–98.

Chakravarti, D., LaMorte, V.J., Nelson, M.C., Nakajima, T., Schulman, I.G., Juguilon, H., Montminy, M., and Evans, R.M. (1996). Role of CBP/P300 in nuclear receptor signalling. Nature *383*, 99–103.

Chen, L., Rao, A., and Harrison, S.C. (1999). Signal integration by transcription-factor assamblies: interactions of NFAT1 and AP-1 on the IL-2 promoter. Cold Spring Harb. Symp. Quant. Biol. *64*, 527–531.

Chen, L., Glover, J.N.M., Hogan, P.G., Rao, A., and Harrison, S.C. (1998a). Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. Nature *392*, 42–48.

Chen, F.E., Huang, D.-B., Chen, Y.-Q., and Chosh, G. (1998b). Crystal structure of p50/p65 heterodimer of transcription factor NF-kB bound to DNA. Nature *391*, 410–413.

Chytil, M., and Verdine, G.L. (1996). The Rel family of eukaryotic transcription factors. Curr. Opin. Struct. Biol. *6*, 91–100.

Descamps-Latscha, B., Herbelin, A., Nguyen, A.T., Roux-Lombard, P., Zingraff, J., Moynot, A., Verger, C., Dahmane, D., de Groote, D., Jungers, P., et al. (1995). Balance between IL-1β, TNF-α, and their specific inhibitors in chronic renal failure and maintenance dialysis. Relationships with activation markers of T cells, B cells, and monocytes. J. Immunol. *154*, 882–892.

Ghosh, G., Van Duyne, G., Ghosh, S., and Sigler, P.B. (1995). Structure of NF-kB p50 homodimer bound to a kB site. Nature *373*, 303–310.

Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. *16*, 225–260.

Goldfeld, A.E., McCaffrey, P.G., Strominger, J.L., and Rao, A. (1993). Identification of a novel Cyclosporin-sensitive element in the human tumor necrosis factor α gene promoter. J. Exp. Med. *178*, 1365–1379.

Goldstein, S., Winston, E., Chung, T.J., Chopra, S., and Pariser, K. (1985). Chronic arthropathy in long-term hemodialysis. Am. J. Med. *78*, 82–86.

Haussinger, D. (1996). The role of cellular hydratation in the regulation of cell function. Biochem. J. *313*, 697–710.

Hedin, K.E., Bell, M.P., Kalli, K.R., Huntoon, C.J., Sharp, B.M., and McKean, D.J. (1997). Delta-opioid receptors expressed by Jurkat T cells enhance IL-2 secretion by increasing AP-1 complexes and activity of the NFAT/AP-1-binding promoter element. J. Immunol. *159*, 5431–5440.

Hoey, T., Sun, Y.L., Williamson, K., and Xu, X. (1995). Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. Immunity *2*, 461–472.

Imler, J.L., and Hoffmann, J.A. (2000). Signaling mechanisms in the antimicrobial host defense of Drosophila. Curr. Opin. Microbiol. *3*, 16–22.

Inglis, A., Hinnie, J., and Kinsella, J. (1995). A metabolic complication of severe burns. Burns *21*, 212–214.

Junger, W., Liu, F.C., Loomis, W.H., and Hoyt, D.B. (1994). Hypertonic saline enhances cellular immune function. Circ. Shock *42*, 190–196.

Junger, W.G., Coimbra, R., Liu, F.C., Herdon-Remelius, C., Junger, W., Junger, H., Loomis, W., Hoyt, D.B., and Altman, A. (1997). Hypertonic saline resuscitation: a tool to modulate immune function in trauma patients? Shock *8*, 235–241.

Kiani, A., Rao, A., and Aramburu, J. (2000). Manipulating immune responses with immunosuppressive agents that target NFAT. Immunity *12*, 359–372.

Kinoshita, S., Su, L., Amano, M., Timmerman, L.A., Kaneshima, H., and Nolan, G.P. (1997). The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. Immunity *6*, 235–244.

Ko, B.C.B., Ruepp, B., Bohren, K.M., Gabbay, K.H., and Chung, S.S.M. (1997). Identification and characterization of multiple osmotic response sequences in the human aldose reductase gene. J. Biol. Chem. *272*, 16431–16437.

Kronfol, N.O. (1994). Acute peritoneal dialysis prescription. In Handbook of Dialysis, J.T. Daugirdas and T.S. Ing, eds. (Boston/New York/Toronto/London: Little, Brown and Company), pp. 301–309.

Kuprash, D.V., Osipovich, O.A., Pokholok, D.K., Alimzhanov, M.B., Biragyn, A., Turetskaya, R.L., and Nedospasov, S.A. (1996). Functional analysis of the lymphotoxin-β promoter. Sequence requirements for PMA activation. J. Immunol. *156*, 2465–2472.

Kwon, H.M., and Handler, J.S. (1995). Cell volume regulated transporters of compatible osmolytes. Curr. Opin. Cell Biol. *7*, 465–471.

Locksley, R.M., Killeen, N., and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell *104*, 487–501.

López-Rodríguez, C., Aramburu, J., Rakeman, A.S., and Rao, A. (1999a). NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. Proc. Natl. Acad. Sci. USA *96*, 7214–7219.

López-Rodríguez, C., Aramburu, J., Rakeman, A.S., Copeland, N.G., Gilbert, D.G., Thomas, S., Disteche, C., Jenkins, N.A., and Rao, A. (1999b). NFAT5: the NFAT family of transcription factors expands in a new direction. Cold Spring Harb. Symp. Quant. Biol. *64*, 517–526.

Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R., and Sigler, P.B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature *352*, 497–505.

Macian, F., and Rao, A. (1999). Reciprocal modulatory interaction between human immunodeficiency virus type 1 Tat and tanscription factor NFAT1. Mol. Cell. Biol. *19*, 3645–3653.

Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., Li, J., Young, D.B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997). IKK-1 and IKK-2: cytokine-activated IkB kinases essential for NF-kB activation. Science *278*, 860–866.

Meyers, A. (1994). Fluid and electrolyte therapy for children. Curr. Opin. Pediatr. *6*, 303–309.

Miyakawa, H., Woo, S.K., Dahl, S.C., Handler, J.S., and Kwon, H.M. (1999). Tonicity-responsive enhancer binding protein, a rel-like protein that stimulates transcription in response to hypertonicity. Proc. Natl. Acad. Sci. USA *96*, 2538–2542.

Müller, C.W., Rey, F.A., Sodeoka, M., Verdine, G.L., and Harrison, S.C. (1995). Structure of the NF-kB p50 homodimer bound to DNA. Nature *373*, 311–317.

Nakanishi, I., Moutabarrik, A., Okada, N., Kitamura, E., Hayashi, A., Syouji, T., Namiki, M., Ishibashi, M., Zaid, D., and Tsubakihara, Y. (1994). Interleukin-8 in chronic renal failure and dialysis patients. Nephrol. Dial. Transplant. *9*, 1435–1442.

Neilson, J., Stankunas, K., and Crabtree, G.R. (2001). Monitoring the duration of antigen-receptor occupancy by calcineurin/glycogen-synthase-kinase-3 control of NF-AT nuclear shuttling. Curr. Opin. Immunol. *13*, 346–350.

Nishikawa, S.I., Hashi, H., Honda, K., Fraser, S., and Yoshida, H. (2000). Inflammation, a prototype for organogenesis of the lymphopoietic/hematopoietic system. Curr. Opin. Immunol. *12*, 342–345.

Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, J.P.B., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P.G., and Rao, A. (2000). Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol. Cell *6*, 539–550.

Parekh, B.S., and Maniatis, T. (1999). Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-β promoter. Mol. Cell *3*, 125–129.

Pereira, B.J.G., Shapiro, L., King, A.J., Falagas, M.E., Strom, J.A., and Dinarello, C.A. (1994). Plasma levels of IL-1β, TNFα, and their specific inhibitors in undialyzed chronic renal failure, CAPD and hemodialysis patients. Kidney Intl. *45*, 890–896.

Rao, A., Luo, C., and Hogan, P.G. (1997). Transcription factors of the NFAT family: regulation and function. Annu. Rev. Immunol. *15*, 707–747.

Ryseck, R.-P., Novotny, J., and Bravo, R. (1995). Characterization of elements determining the dimerization properties of RelB and p50. Mol. Cell. Biol. *15*, 3100–3109.

Screaton, G., and Xu, X.N. (2000). T cell life and death signalling via TNF-receptor family members. Curr. Opin. Immunol. *12*, 316–322.

Sengchanthalangsy, L.L., Datta, S., Huang, D.B., Anderson, E., Braswell, E.H., and Ghosh, G. (1999). Characterization of the dimer interface of transcription factor NF-kB p50 homodimer. J. Mol. Biol. *289*, 1029–1040.

Shapiro, L., and Dinarello, C.A. (1995). Osmotic regulation of cytokine synthesis in vitro. Proc. Natl. Acad. Sci. USA *92*, 12230–12234.

Shapiro, L., and Dinarello, C.A. (1997). Hyperosmotic stress as a stimulant for proinflammatory cytokine production. Exp. Cell Res. *231*, 354–362.

Smolen, J.S., Tohidast-Akrad, M., Gal, A., Kunaver, M., Eberl, G., Zenz, P., Falus, A., and Steiner, G. (1996). The role of T-lymphocytes and cytokines in rheumatoid arthritis. Scand. J. Rheumatol. *25*, 1–4.

Takayama, F., Miyazaki, T., Aoyama, I., Tsukushi, S., Sato, M., Yamazaki, C., Shimokata, K., and Niwa, T. (1998). Involvement of interleukin-8 in dialysis-related arthritis. Kidney Intl. *54*, 1007–1013.

Trama, J., Lu, Q., Hawley, R.G., and Ho, S.N. (2000). The NFAT-related portein NFATL1 (TonEBP-NFAT5) is induced upon T cell activation in a calcineurin-dependent manner. J. Immunol. *165*, 4884–4894.

Wesselborg, S., Bauer, M.K.A., Vogt, M., Schmitz, M.L., and Schulze-Osthoff, K. (1997). Activation of transcription factor NF-kB and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. J. Biol. Chem. *272*, 12422–12429.

Zhou, P., Sun, L.J., Dötsch, V., Wagner, G., and Verdine, G.L. (1998). Solution structure of the core NFATC1/DNA complex. Cell *92*, 687–696.